DEVELOPMENT OF MONOSPECIFIC ANTI-BEEF SERA

By

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THESIS

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Department of Veterinary Public Health

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MANNUTHY, THRISSUR

KERALA

1995

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I hereby declare that the thesis entitled **DEVELOPMENT OF MONOSPECIFIC ANTI BEEF SERA** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society

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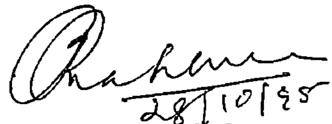

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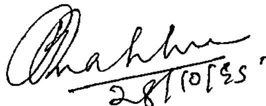

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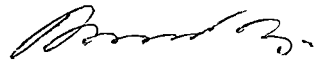

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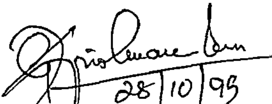

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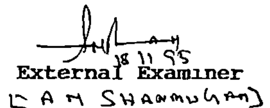

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LIST OF ABBREVIATIONS (KEY WORDS)

AGID	- Agar gel immuno-difusion
BFD	- Freeze dried buffalo serum
BME	- Buffalo meat extract
CaME	- Camel meat extract
CFD	- Freeze dried cattle serum
CME	- Cattle meat extract
DME	- Deer meat extract
FD-BME	- Freeze dried buffalo meat extract
FD-GME	- Freeze dried goat meat extract
GFD	- Freeze dried goat serum
GME	- Goat meat extract
IE	- Immuno - electrophoresis
PBS	- Phosphate buffered saline
PBS-T	- Phosphate buffered saline tween - 80
PME	- Pig meat extract
RABS	- Rabbit anti-buffalo serum
RABS(M)	- RABS against FD-BME in standardization of the experiment
RABS(S)	- RABS against buffalo serum in standardization of the experiment
RACS	- Rabbit anti-cattle serum
RACS(A)	- RACS from group A in standardization of the experiment (i e , against normal serum)
RACS(B)	- RACS from group B in standardization of the experiment (i e , against heated serum)
SFD	- Freeze Dried Sheep Serum

Introduction

INTRODUCTION

India occupies a unique position in the field of livestock production in the world At present, 15, 20 9 and 50 per cent of cattle goat and buffalo population respectively of the world are found in India The annual meat production in the country is estimated to be about 4 44 million tons valued at about Rs 6000 million Even though about 67 per cent of the Indian subjects are reported to be non-vegetarians, the per capita consumption of meat is only 5 11 kg (14 g / day per head) which is far below the ICMR recommended quantum of 12 41 kg (34 g / day per head)

With the changing food habits and increased awareness of the nutritional requirements, there is an increasing demand for meat and meat products At this juncture the present time can be considered as a period of gourmets and gourmands The preferential demand for meat and meat products is influenced by many factors, like religious sentiments taboo cultural habits, customs, availability and prevailing price per unit

In some states of India slaughter of cow is prohibited by the law and export of beef from the country is totally banned In contrast there is no restriction for slaughter of buffalo and export Generally beef is available in plenty in many parts of the country as against buffalo meat and is

cheaper to chevon, mutton, pork and chicken Among meat consumers many prefer buffalo meat Fraudulent substitution and adulteration of meat, particularly highly priced ones with cheaper ones, has been a subject of global concern In India, it has been estimated that 25-30 per cent of the market meats are adulterated Substitution of beef for buffalo meat is suspected in the meat export trade and local markets The main reasons for the substitution or adulteration of buffalo meat with beef are its suitability for clandestine export, the higher demand of buffalo meat by some local consumers and, its resemblance to buffalo meat in physico-chemical properties and organoleptic characters

Prevention of adulteration and fraudulent substitution of meat and meat products is a must for the following reasons -

(i) To prevent economic loss,

(ii) to comply with religious sentiments and dietary restrictions,

(iii) to comply with national laws,

(iv) to prevent the spread of certain diseases, and

(v) to quantify the various types of meat in meat products

Considering all these facts, identification of species of origin of meat is of considerable importance in veterinary forensic medicine and in quality control of meat and meat products

The various methods available at present for speciation of meat are as follows -

1 **Immunological techniques** comprising agar gel immunodiffusion (AGID), immunoelectrophoresis (IE) counter immunoelectrophoresis (CIE), enzyme - linked immunosorbent assay (ELISA) and immunonephelometric assay

2 **Eletrophoretic techniques** such as starch gel electrophoresis (STAGE), polyacrylamide gel electrophoresis (PAGE), sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), polyacrylamide gel isoelectric focusing (PAGIF) and agarose gel isoelectric focusing (AGIF)

3 **Other techniques** like fatty acid pattern, histidine dipeptides direct probe mass spectrometry, DNA hybridization assay and polymerase chain reaction (PCR)

The criteria for considering any of these tests as an ideal one are its ability to (a) detect a wide range of species, (b) differentiate closely related species, (c)

detect low levels of adulteration, (d) to give speedy results and (e) cost effective

To date, all the methods now in vogue have their own merits and demerits and no test can be singled out as an ideal one However sensitivity, simplicity, economical and technical feasibility have made the immunological methods more attractive The main disadvantage is the cross-reaction between phylogenetically closely related species To circumvent this practical problem, the development of monospecific antisera to each species to be identified is of prime importance and priority Up till now, this effort has only been met with different degree of success Hence, the present study is an effort to evolve a suitable method to develop monospecific anti-beef sera The anti-beef sera is to be raised in rabbits using fresh serum as immunogen and the cross-reacting antibody is to be removed by absorption with antigens from closely related species to differentiate between beef and buffalo meat by Agar Gel Double Immunodiffusion tests

A major problem encountered by the field veterinarian, in connection with meat speciation, is the transport of meat from remote area to laboratory without losing its antigenicity An attempt is also to be made herein to study the suitability of filter paper as a carrier for meat antigen from the field

Review of Literature

REVIEW OF LITERATURE

Misrepresentation and fraudulent substitution of meat and meat products are prevalent in many countries of the world Such fraudulent practices have been reported by various workers (Katsube and Imaizumi, 1968, Kang ethe et al 1982 Whittaker et al 1983 King 1984 Cutrufelli et al , 1991, Sherikar et al , 1994) In India, it was reported that about 25-30 per cent of meat in the market is adulterated or substituted (Jacob, 1976) Recent investigation in Bombay revealed that 41 66 per cent of Buffalo beef samples were substituted with beef and about 33 33 per cent of beef with buffalo meat (Sherikar et al 1994)

Attempts have been made in India and abroad by various workers to develop a simple but reliable and inexpensive method of identifying species of origin of meat The main problem of identifying phylogenetically related species is the strong cross-reaction of the antisera with the heterologous species antigen In order to overcome this problem and to enhance sensitivity of the test, use of monospecific antiserum has been recommended (Pinto, 1961, Swart and Wilks 1982, Kang ethe et al , 1986, Bansal and Mandokhot, 1988)

2 1 Production of hyperimmune serum

2 1 1 The Host Animals

Various experimental animals had been used for production of hyperimmune sera Rabbits were most frequently used by various workers due to various reasons Certain workers have recommended the Rabbits as suitable host for raising hyperimmune sera (Evans, 1957, Boyd, 1966, Chase, 1967 Kwapinski, 1972 Crowle, 1973 Somasekharan 1983, Nanu et al , 1985) But the use of rabbits as serum raising animal has certain disadvantages in that there is great individual variation in the development of antibodies even within the same breeds and hence at least a batch of five rabbits has to be immunised at a time (Chase, 1967, Shaw et al , 1983, Bansal and Mandokhot 1988 a)

Other small animals used for raising hyperimmune serum include guinea pigs rats, mice, hamsters and chicken (Chase, 1967, Kwapinski, 1972, Crowle, 1973) Though they may yield antiserum of good titre, they are not routinely used since they yield very little serum (Kwapinski, 1972)

Large animals like cattle, buffalo, horse, goat, sheep and monkey have been used for raising antiserum (Chase, 1967, Kwapinski, 1972, Crowle, 1973, Kang ethe et al 1986, Reddy, 1990) Animals such as Horse, Cattle, Sheep, Goat and Buffalo are mainly used to produce large quantities of

serum in commercial enterprises (Kwapinski 1972, Chase, 1967) However, the use of large animal for raising antiserum is recommended for the production of monospecific antisera against closely related animals (i e by cross-immunization) (Pandey and Pathak 1975 Patterson and Spencer 1985, Bansal and Mandokhot, 1988a, Srinivas, 1991) The main drawback in their use is that a comparatively longer immunization period and large quantity of immunogen are required to develop antibody (antiserum) of high potency and therefore, it is also time consuming (Chase, 1967)

2 1 2 Immunogens, Adjuvants and Immunization

The selection of host animals, type and quality of immunogens, route of injections are all important factors for production of antisera (Proom, 1943 , Warnecke and Saffle 1968 Kwapinski 1972 and Somasekharan, 1983)

Pinto (1961) used alcohol precipitated protein fraction of blood sera as immunogen for production of hyperimmune serum in rabbit and obtained antiserum of very high titre Bubloz (1962) was able to produce immune serum in rabbit using meat extract and blood serum of horse, ox, sheep and dog Soetarjo (1964) developed antisera of fairly high titre in rabbit by injecting alum precipitated serum and whole serum Katsube and Imaizumi (1968) used thermal in-activated serum (56°C for 30 min), boiled serum, autoclaved serum raw meat extract autoclaved meat extract of horse,

cow and whale as immunogens Alum was used as adjuvant to all serum immunogens and alum plus incomplete Freund s adjuvants in meat extracts were administered intramuscularly It was observed that serum injected rabbits yielded antiserum of high titre than those of meat extracts Moreover, autoclaved meat extract did not yield detectable precipitates Warnecke and Saffle (1968) had tried actomyosin, serum, serum alum precipitate, water muscle extract alum precipitate saline extract of skeletal muscle and freeze dried water extract of skeletal muscle They observed that intramuscular injections of freeze dried water extract of skeletal muscle with adjuvant was superior to the others Helm <u>et al</u> (1971) used freeze dried water-soluble extract of skeletal muscle rehydrated in saline @ 150 mg / 2 ml saline and emulsified in 1 5 ml of Freund s complete adjuvant and injected intramuscularly for two consecutive days, repeating the injection for another two-consecutive days, namely on 22nd and 23rd day All the rabbits produced antiserum of good titre except two rabbits injected with beef and pork muscle extract Shanmugam and Ranganathan (1972) produced hyperimmune sera in Rabbit against mutton and beef by intraperitoneal injection of - (a) mutton aqueous extract plus sheep serum and (b) beef aqueous extract plus bovine serum and found effective against the respective, homologous meat extract Ramadass and Misra (1981) produced immune sera by injecting the

rabbits with alum precipitated muscle extract intramuscularly

Whittaker et al (1983) and Patterson et al (1984) inoculated the rabbits using serum protein (pooled from at least three animals) with Freund s complete adjuvant initially and booster dose with Freund s incomplete adjuvant When the animals were bled 8-10 days after the last injection they could get antisera of good titre In the comparative study on the efficacy of different antigens and route of administration for anti-beef sera production Nanu et al (1985) observed that blood serum was the choice antigen for production of anti-beef sera in rabbit compared to saline extracts of meat and bone marrow and they also observed that intramuscular route was preferable

Bansal and Mandokhot (1988 a) raised anti-beef sera in rabbit and buffalo calves using freeze dried skeletal muscle extract of cattle Many workers have developed antisera specific to thermostable muscle protein (TMP) by immunizing the host with TMP or adrenal BE (Boiling resistant ethanol precipitate) (Kang ethe et al 1986 Radhakrishna et al 1988 Sherikar et al (1988 1993) Karkare et al (1988) and Reddy et al 1990) Srinivas et al (1991) raised antisera in rabbit using fresh liver extract of sheep cattle and buffalo as immunogen but the antisera so produced could not be made monospecific

2 1 3 Harvesting the antiserum

Pinto (1961) suggested starvation of the rabbits for the last 24 h before terminal bleeding The rabbits were then bled and the blood was allowed to clot for one hour, the clotted blood was ringed with sterile glass rod and left a few more hours at room temperature and then at 4°C for overnight The serum was collected, centrifuged and stored in refrigerator According to Chase (1967), harvesting the immune serum is done by ear scarification on the fifth or sixth day from the last injection and fluid balance is restored by injecting equal volume of physiological saline intraperitoneally Exsanguination is practised two to four days later He also suggested withholding of food but not water for one night prior to bleeding Karpas et al (1970) practised collection of blood by cardiac puncture one weak after last injection Collection of blood by cardiac puncture after 21 days of the last injection has been reported by Helm et al , (1971) Kwapinski (1972) also recommended withholding of feed for the last 24 h prior to bleeding to avoid accumulation of lipids in serum in high concentration He also stated that final bleeding performed at 5th to 7th day after last injection yielded maximum antibody titre Patterson et al (1984) bled the immunised rabbit 8-10 days after the last dose from the jugular vein Radhakrishna et al (1988) also practised bleeding the animal 10 days after the last dose and repeated on the 15th day

2 1 4 Preservation and storage of antiserum

According to Chase (1967), Sterile immune serum of good initial titre can be held safely at 4°C for a long period of time Sterilization is done by filtering the serum through cellulose acetate filters i e Millipore Type G S 0 22 micrometre average pore size He also recommended the use of preservatives such as 1 10,000 merthiolate (Thiomersal 1 per cent of 1 per cent stock solution), 0 1 per cent sodium azide (1 per cent of 10 per cent stock), and 1 10 000 8-hydroxquinoline sulfate (8-quinolinol sulfate) Preservation by freezing and storage at -16°C in small lots and lyophilization also can be employed

Katsube and Imaizumi (1968) reported that the antiserum from the rabbit was inactivated at 56°C for 30 min and preserved by the addition of thiomersal to the final concentration of 1 5000 Kwapinski (1972) stated that serum harvested from rabbits can be stored at -30°C for many months or for years and, for longer storage, frozen serum is lyophilised Crowle (1973) stated that the three most common means of storing antiserum are by refrigeration at 4°C, by freezing and by lyophilization. Hayden (1981) reported that the harvested antisera was freeze dried in 2 ml lots and stored at -20°C and aliquots were reconstituted with one ml distilled water to increase the antibody content per unit volume Kang ethe _et al_ (1986) also reported

storage of the serum at -20°C Sherikar et al (1987 a) preserved the serum by distributing in 5 ml screw capped glass tubes and thiomersal added at the final concentration of 1 10000 and stored at -20°C Bansal and Mandokhot (1988 a) pooled the antisera from different host individuals and preserved with 1 10000 thiomersal The pooled sera was distributed in 8-10 ml lots, heated in water bath at 56°C for 30 min, centrifuged at 5000 rpm and supernatant stored at -20°C till used for different immunological tests

2 2 Immunoabsorption

Antiserum raised in phylogenetically distant animals usually contain antibodies which cross-react with many of heterologous species antigens For instance, antiserum raised in rabbits against cattle cross-react non specifically with antigens from Buffalo, Sheep and Goat This type of cross-reactions have been reported by many workers (Pinto, 1961, Soetarjo, 1964, Warnecke and Saffle, 1968, Karpas et al , 1970 Pandey and Pathak, 1975 Swart and Wilks, 1982, Tizard et al , 1982, Somasekharan, 1983, Ramadass and Misra, 1983, Kang'ethe et al ,1985, Sherikar et al , 1987b, 1988, Bansal and Mandokhot, 1988a, Radhakrishna et al , 1988, Srinivas et al 1991 and Aulakh et al , 1994) Probable reasons for cross-reactions occuring between closely and distantly related species of animals

has been discussed by Proom (1943) Weitz (1952), Omland (1963a), Muraschi et al , (1965), Rodkey and Freeman (1970), Esteves and Binaghi (1972) and Batty (1984)

Successful immunoabsorption has been reported by Pinto (1961) using dilution of 1/200 of normal ox, buffalo, goat and deer blood sera Avrameas and Ternynck (1967) used copolymerised water insoluble protein for absorption and removal of cross-reacting antibodies Warnecke and Saffle (1968) used freeze dried antigenic protein of the species which were cross-reacting for absorption But in many instances after absorption the antibody titre became so low that it could not react even with homologous antigen, but, when antibody titre was very high, the problem was overcome and monospecific antisera could be obtained successfully

According to Karpas et al (1970), species cross-reactive antisera were made monospecific by absorption with purified heterologous IgG (Immunoglobulin G) precipitates However, by this method all the antisera absorbed cannot be made monospecific The number and types of absorption with heated IgG precipitates required to render an antiserum monospecific varied and had to be determined for each individual antiserum Hayden (1979) failed to remove cross-reacting antibodies from antiserum to lamb myoglobin by immunoabsorption with bovine myoglobin coupled to an

insoluble agarose matrix and by absorption with fresh ground beef The remaining antibody titre was too low to be effective in precipitin analyses However immunoabsorption with freeze dried aqueous extract of muscle has been successful Swart and Wilks (1982) outlined the method of removing cross-reacting antibodies by absorption with relevant species proteins However, they could not differentiate between species of closely related animals such as cattle and buffalo Doberstein and Greuel (1985) reported successful elimination of cross-reacting antibodies present in antisera to springbok and impala raised in rabbits, by absorption with heterologous blood sera, but not of anti-eland sera which still reacted with the meat extract of closely related kudu Kang ethe et al (1986) succeeded in production of species-specific antisera to thermostable muscle antigen (TMA) of buffalo, eland, water buck, topi, wildebeest and oryx using absorption method of Avrameas and Ternynck (1967), in which water insoluble protein polymer from 14-heterologous species was used But antisera to cattle TMA could not be made species-specific as it still cross-reacted with fresh meat antigen (FMA) of buffalo Similarly, antisera to TMA of Grant s gazelle, kongoni, Thomson s gazelle and topi could not be made monospecific

Bansal and Mandokhot (1988 a) have reported successful development of monospecific anti-beef sera In their study they raised anti-beef sera in rabbits against freeze dried

skeletal muscle extract of cattle and the antisera being absorbed with the mixture of freeze dried skeletal muscle extract of buffalo, sheep and goat Sherikar *et al* (1988) reported successful immunoabsorption and development of species-specific antisera to adrenal BE antigens of cattle, buffalo, sheep goat and pig The antisera to adrenal BE of cattle buffalo sheep goat and pig were absorbed with BE antigens of buffalo, cattle, goat, sheep and sheep respectively Radhakrishna *et al* (1988) produced antisera to thermostable meat protein of ox and buffalo in sheep but could not remove the cross-reacting antibodies between the two species by absorption with TMP of sheep and goat

2 3 Techniques for Species Identification of Meat

Various techniques have been developed for the identification of meat species Broadly they are physical, chemical, immunological and electrophoretic methods According to Kurth and Shaw (1983) and Wijngaards and van Biert (1985), immunological and electrophoretic techniques are more common and useful Physical and chemical methods have been reviewed in detail by Sharma *et al* (1986)

2 3 1 Immunological methods

Immunoassays, based on very specific antigen-antibody interaction, can detect and determine particular analytes *in situ* in complex mixture such as biological fluids or food

extracts While there are many analytical procedures in which immunoreagents can be used, the two most useful technique for application in species recognition are the classical Ouchterlony double immunodiffusion technique and the more recent enzyme-linked immunosorbent assay (ELISA) (Hitchcock and Crimes, 1985; Smith, 1995)

2 3 1 1 Single diffusion test

Single diffusion test is done in capillary tube, in which the antigen is layered over the antiserum This test has been used for species identification of meat by certain workers (Pinto 1961, Warnecke and Saffle, 1968, Katsube and Imaizumi, 1968) The main advantage of single diffusion test is its simplicity to perform and obtain the result within few minutes The disadvantages are, difficulty in reading the result diffusion of the resultant lines within a short period of time and their disappearance, false positive reaction and difficulty in making permanent record of the result (Swart and Wilks, 1982, Kurth and Shaw, 1983)

2 3 1 2 Agar Gel Immunodiffusion (AGID) or, The Classical Ouchterlony Double Immunodiffusion test

Immunodiffusion tests in which both antigen and antibody diffuse towards each other in an inert medium and react forming a precipitin line are known as double diffusion tests (Crowle, 1973) Bubloz, (1962) reported

detection of one part of horse meat in nine parts of beef by double diffusion method Soetarjo (1964) reported that anti-horse anti-pig and anti-dog sera prepared by Proom s method showed high specificity in gel diffusion test, forming precipitation lines with the homologous meat only However, he observed cross-precipitation between beef, mutton, buffalo and goat meat and their antisera Tagore _et al_ (1977) reported the successful identification of cattle and buffalo from other animals by immuno-double diffusion test using species-specific antisera absorbed by the method of Pinto (1961) Hayden (1978) detected the presence of 1 3 and 5 per cent flesh of pig, horse and rabbit in fresh ground beef by agar gel precipitation test Sherikar _et al_ (1979) used Ouchterlony s double gel diffusion test for differentiation of meat from cattle, buffalo, sheep, goat, pig and poultry In an immunodiffusion method developed by Swart and Wilks (1982), the detectable level of horse meat kangaroo meat and mutton, when mixed with beef, were 5, 20 and 20 per cent respectively Shaw _et al_ (1983) reported the detection level of two per cent for kangaroo meat in beef or mutton using immunodiffusion method developed by them Kang ethe _et al_ (1985) reported successful development of monospecific antisera to thermostable muscle antigens, from 13 wild animals and seven domestic animals, by absorption with copolymerized pooled serum from the 20 - species and thermostable muscle antigens With this

monospecific antisera, they could identify the species origin of saline extracts of both cooked and fresh meat samples in immuno-diffusion test Karkare et al (1988) produced monospecific antisera to adrenal BE antigen of buffalo cattle, sheep and goat by absorbing anti buffalo sera with cattle BE antigen, anti-cattle sera with buffalo BE antigen, anti-sheep sera with goat BE antigen and, anti-goat and anti-pig sera with sheep BE antigens The antisera were tested by means of double immunodiffusion method Bansal and Mandokhot (1988 b), in their comparative study, came to the conclusion that double immuno-diffusion (DID) test was most suitable and ideal from the point of simplicity where laboratory facilities were elementary and antiserum supply was limited when compared to counter current immunoelectrophoresis (CIE) and immunoelectrophoresis (IE) Wintero et al (1990) reported that the detectable level of pork in beef was one per cent using immunodiffusion test

Field tests, based on AGID technique, Overnight Rapid Bovine Identification test (ORBIT) and Poultry Rapid Overnight Field Identification Test (PROFIT), were developed by Mageau et al (1984) and Cutrufelli et al (1986) and subsequently had been approved by Association of Official Analytical Chemist (AOAC, 1990) The detectable level by ORBIT and PROFIT is $\geq$ 10 per cent, when a product is adulterated with beef or chicken (Cutrufelli et al ,

1987) Similar species identfication field tests that have been developed are Porcine Rapid Identification Method (PRIME) for pork Serological Ovine Field Test (SOFT) for sheep , Rapid Equine Serological Test (REST) for horse and Deer Rapid Identification Field Test (DRIFT) for deer (Cutrufelli <u>et</u> <u>al</u> , 1988, 1989, 1991 and 1992)

2 3 1 3 Immunoelectrophoresis

Electrophoresis can be defined as the movement of charged particles or ions from one location to another by direct current through an electrolyte solution (Crowle, 1973 Arguembourg, 1975) Immunoelectrophoresis (IE) is the combination of electrophoresis and specific precipitation by double gel diffusion (Carpenter, 1975) The pH of the buffer (the electrolyte) determines the direction of reactant movement, reactants with isoelectric point above the pH of the buffer tend to move towards the cathode (negative pole) those with lower isoelectric point are repelled by the cathode and move towards anode (positive pole) and those with the same isoelectric point as the pH of the buffer remain electrophoretically immobile (Crowle, 1973) In IE electrophoretic run of the antigen is carried out first and then immunodiffusion with the antiserum

Ramadass and Misra (1981) reported that muscle antigens of bullock, buffalo, goat, sheep, pig and chicken could be

differentiated by means of IE as per the number and location of precipitin arcs in the immunoelectrophoretogram Casa et al (1985) observed that the IE is more sensitive and possibility of getting false positive is less, when compared to Ouchterlony immunodiffusion test According to them the cost of IE in agarose gel for detection of meat adulteration in large scale will be the main disadvantage of this technique Srinivas et al (1991) could not differentiate between sheep, goat cattle and buffalo by IE, when antisera to fresh liver antigen of these species were raised in rabbit and tested against the muscle extract of homologous and hetrologous species Aulakh et al (1994) reported the differentiation of meats of cattle, buffalo, goat, sheep, pig and poultry on the basis of species specific number and position of precipitin arcs

2.3 1 4 Counter current Immuno-electrophoresis (CIE)

This is an accelerated immunodiffusion in which the diffusion of both antigen and antibody (antiserum) are assisted by the application of an electric charge and is particularly effective where speed and cost are important factors (Hitchcock and Crimes, 1985, Ansfield, 1985, Allsup, 1987, Bansal and Mandokhot, 1988, Wintero et al , 1990, Sherikar et al , 1994)

2 3 1 5 Enzyme Linked Immunosorbent Assay (ELISA)

Unlike Immunodiffusion test, ELISA does not rely on the precipitation of the antigen-antibody complex but here antigen-antibody interaction occurs in a monomolecular layer immobilised on an inert surface and is followed by means of an enzyme chemically bonded to one of the immunoreagents (Hitchicocks and Crimes 1985) Therefore in ELISA the presence of the antigen-antibody complex is quantitatively monitored by colorimetric measurement of the activity of the enzyme linked to it (Olsman et al , 1985) Various versions of ELISA techniques such as indirect ELISA (Kang ethe et al , 1982, Whittaker et al , 1983), improved double sandwich ELISA (Patterson et al , 1984) modified indirect ELISA (Jones and Patterson, 1986), Comparative ELISA (Dincer et al 1987), Sandwich ELISA (Martin et al , 1988, 1991) and immunometric ELISA (Govindarajulu, 1994) have been used for speciation of meat and found very effective and useful in terms of sensitivity, speed and economy in large scale screening One ml of antiserum would be sufficient for 1000 assays or more (Hitchcock et al 1981 Whittaker et al 1983, Patterson and Spencer, 1985) The main disadvantages are its requirement of expensive reading equipment and monospecific antisera (Kurth and Shaw, 1983, Patterson and Spencer, 1985, Hitchcock and Crimes, 1985)

2 3 2 Electrophoretic Techniques

Electrophoretic methods achieve the separation of proteins by their differential migration through a supporting medium under the influence of an electric field Supporting gels which may be homogenous gel concentration gradient gels pH gradient gels or denaturants (such as urea or detergents that dissociate the tertiary protein structures) govern the separation of proteins These proteins can be visualised by simple non specific stain or by enzymological or immunological methods Protein patterns can then be compared by direct observation or after densitometric scanning (Kurth and Shaw, 1983, Hitchcock and Crimes, 1985)

Starch gel electrophoresis (STAGE) has been used for identification of species origin of meat (Thompson, 1961 1968, Prasad and Misra, 1981, Yman and Sandberg, 1987) Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were reported to be more superior electrophoretic techniques than that of STAGE and was used for speciation of meat by many workers (Payne, 1963, Hoyem and Thorson, 1970, Babiker et al , 1981, Bandyopadhyay et al , 1985, Carnegie et al , 1985 Heinert et al , 1988)

Isoelectric focusing in polyacrylamide gel (PAGIF) and in agarose gel (AGIF) are useful techniques to differentiate

the meat species, by observing species specific protein band pattern of meat extract It has been used successfully by certain workers to identify the species origin of meat including closely related species (Kaiser et al , 1980, Abraham 1982 Hamilton, 1982 King and Kurth, 1982 Slattery and Sinclair, 1983, King, 1984, Ansfield, 1985, Vizzani et al 1985 Yman and Sandberg 1987 Abraham and Rajulu 1992, McCormick et al , 1992, Govindarajulu, 1994)

It is certain that, electrophoretic techniques have some advantages over immunological methods, for instance, their ability to identify between closely related species in raw meat samples and they do not require antisera (Abraham, 1982 Grundhofer 1985) However the limitation are also many, including expensive chemical reagents and equipments, requirement of technical expertise to perform the test and interpret the result, and a relatively high cost factor per sample analysed Therefore use of such method is prohibitive in large scale testing (Mageau et al , 1984, Hitchcock and Crimes, 1985 Wintero et al , 1990)

2 3 3 Other useful methods for meat speciation

During the last few decades, some workers in the field of meat speciation have tried different techniques with promising results but not without limitations These are Radio-immunoassay (RIA) (Johnston et al , (1982), Histidine

dipeptides estimation by high performance liquid chromatography (HPLC) (Carnegie et al 1984, 1985), Direct probe mass spectrometry (Puckey and Jones, 1984), DNA hybridization (Chikuni et al , 1990, Wintero et al , 1990, Ebbehoj and Thomsen 1991a and 1991b), Unlabelled antibody peroxidase anti-peroxidase (PAP) technique (Karkare et al , 1989 Sherikar et al , 1993) and Polymerase Chain Reaction (PCR) technique (Chikuni et al , 1994, Nachimuthu, 1994 and Appa Rao et al , 1995)

MATERIALS AND METHODS

3 1 Standardization of the experiment

For the production of antisera, six young adult New Zealand white rabbits were procured from Small Animal Breeding Station, Mannuthy Rabbits were randomly divided into three groups namely A , B and C , comprising two in each group Group A were immunised with cattle serum Initially 0 5 ml of serum was injected to each rabbit intramuscularly and 1 ml in subsequent injections at an interval of five-days A total of 10-injections were given to individual rabbits during the course of immunization Group B were subjected to immunization with heat treated cattle serum (at 56°C for 30 min in water bath) following the same dose and route as that of Group A One of the rabbits in Group C was immunized with buffalo serum by the same procedure as that of Group A The other rabbit was immunised with freeze dried buffalo meat extract reconstituted in physiological saline (2 ml buffalo meat extract is reconstituted in 2 ml sterile physiological saline) The dose and route of administration were the same as that of Group A

To assess the development of antibody and the effect of number of injection in producing cross reacting antibodies, the rabbits were test bled just before 5th

injection and the sera of individual rabbits were tested against the homologous as well as heterologous antigens by Agar Gel Immuno-diffusion (AGID) test Terminal bleeding was carried out by ear scarification, followed by severing the jugular vein on the 10th day after the last injection About 20 ml of blood was collected from each animal and the sera obtained within groups A and B were pooled together whereas in group C pooling of sera was not done To all sera, merthiolate was added to the final strength of 1 10,000 and stored at 4°C

Sera from Group A and B were divided into four parts the first part being kept as such (unabsorbed) The other three parts were subjected to absorption with freeze dried goat serum (GFD) freeze dried sheep serum (SFD), and freeze dried buffalo serum (BFD) respectively The sera from individual rabbits of Group C were absorbed in the same manner except for the fact that instead of BFD, freeze dried cattle serum (CFD) was used The absorbed and unabsorbed antisera from all the groups were tested against fresh serum and meat extract of cattle, buffalo and goat by means of AGID

3 2 Experiment Proper

3 2 1 The Rabbits

Six young adult New Zealand white rabbits were procured from Small Animal Breeding Station, Mannuthy They were fed

with standard concentrate feeds @ 100g/animal/day and greens *ad libitum* After acclimatization to the new environment, they were randomly divided into Group A and B comprising three in each group

3 2 2 The immunogens

For immunizing the rabbits,sera of cattle and buffalo were collected aseptically, each time before immunization schedule Pooled cattle serum and buffalo serum were used for immunization

3 2 3 Immunization

Rabbits in group A were immunized with fresh cattle serum and that of Group B were immunized with fresh buffalo serum following the method described in the standardization of the experiment with the exception that only eight injections were given to each rabbit

3 2 4 Test bleeding of Rabbits

To assess the development of antibody the test bleeding of rabbits were performed before the 5th injection The antisera obtained was tested against homologous as well as heterologous antigens by AGID

3 2 5 Terminal bleeding

Terminal bleeding of the rabbits was done on the 10th day from the last injection, initially by ear scarification

and then by severing the jugular vein Blood from each rabbit was collected in a sterile 60 ml test tube and allowed to clot in slanting position at room temperature After 6 h it was kept at 4°C overnight

The sera were collected in sterile test tubes, centrifuged and the clear serum obtained from individual rabbits within a group was pooled together and merthiolate was added to the final strength of 1 10,000 They were then stored at 4°C

3 2 6 Immunoabsorbents

For removal of cross reacting antibodies present in the antisera immunoabsorbents were prepared by freeze drying fresh whole sera of cattle (CFD), buffalo (BFD), sheep (SFD) and goat (GFD) The sera collected from each species were distributed as 2 ml in each sterile vial and freeze dried at Institute of Animal Health and Veterinary Biologicals, Palode The freeze dried sera were stored at 4°C

3 2 7 Immunoabsorption of Antisera

The antisera raised against cattle and buffalo were made monospecific using the technique described by Bansal and Mandokhot (1988a) with the following modifications -

Antisera from Group A rabbits were divided into four parts First part was stored as unabsorbed antiserum

(control) The second part was absorbed with GFD @ 250 mg / 2 ml Rabbit Anti-cattle serum (RACS) The third part was absorbed with BFD @ 250 mg / 2 ml RACS The fourth part was subjected to absorption by mixing with a combination of 125 mg GFD and 125 mg BFD / 2 ml RACS Antisera from group B rabbits were processed and absorbed in the same manner as that of RACS except for the fact that the third part of Rabbit Anti Buffalo Serum (RABS) was absorbed with CFD and fourth part with the mixture of GFD and CFD

3.2.8 Meat samples

Cattle and buffalo meat samples to be tested were procured from various retail outlets of Trichur town as well as from University sales counter, Mannuthy

3 2 9 Preparation of Antigens

To prepare test antigens 50 gms of meat samples from each species of animal was taken and cut into small pieces and then minced in a mixer-cum-grinder The minced meat was wrapped with muslin cloth and pressed hard The extract was collected directly into a clean test tube and centrifuged for 20 min at 5000 rpm The supernatant was collected in clean vials and used for testing

To evaluate the lowest level of adulteration detectable in binary meat mixtures, test meat antigens containing 20,

25 50, 75 and 80 per cent beef in buffalo beef and buffalo beef in beef were used with RACS and RABS in AGID

Antigens prepared from a total of 30 beef (CME) and 30 buffalo beef (BME) samples, two deer meat samples (DME) one pig meat sample (PME) and one camel meat sample (CaME) have been used for various tests Ten samples of meat which were given for identifying their species of origin also formed the materials in the experiment

3 2 10 Agar Gel Immuno-diffusion Test

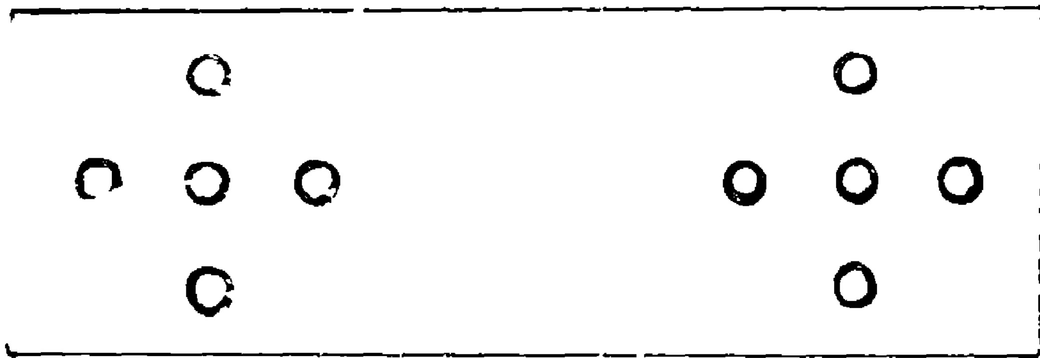
Both absorbed and unabsorbed antisera obtained from group A and B rabbits were subjected to AGID against homologous and heterologous antigens For this purpose 0 65% agarose in 0 85% sodium chloride solution was prepared on agarose pre-coated microscope slide (2 5 x 7 5 cm) with 3 ml molten agarose gel per slide After proper solidification and cooling of gel, 2 - sets of wells were made as per the details shown in Fig 1 The particulars of the test programmes were as follows both absorbed and unabsorbed antisera were tested against fresh meat antigen, freeze dried serum amd freeze dried meat extract obtained from goat, cattle and buffalo and other meat antigens as stated under 3 2 9 The peripheral wells were charged with test antigens approximately with 22 microlitre per well using capillary tube and the central well with 22 microlitre of the required antisera The slides were then

Fig. 1 Patterns of making wells on the agarose gel slide

A Four peripheral wells and a central well

B Two peripheral wells and a middle one

Internal diameter of each well is 3 mm, the distance from peripheral well to central/middle well (edge to edge) is [illegible] mm



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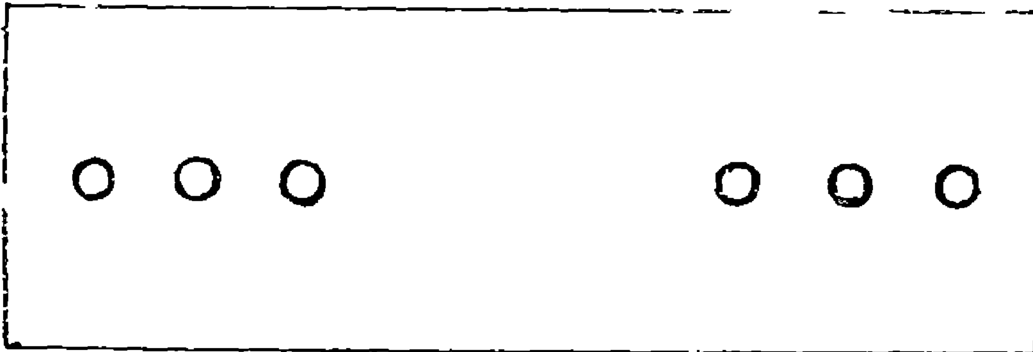
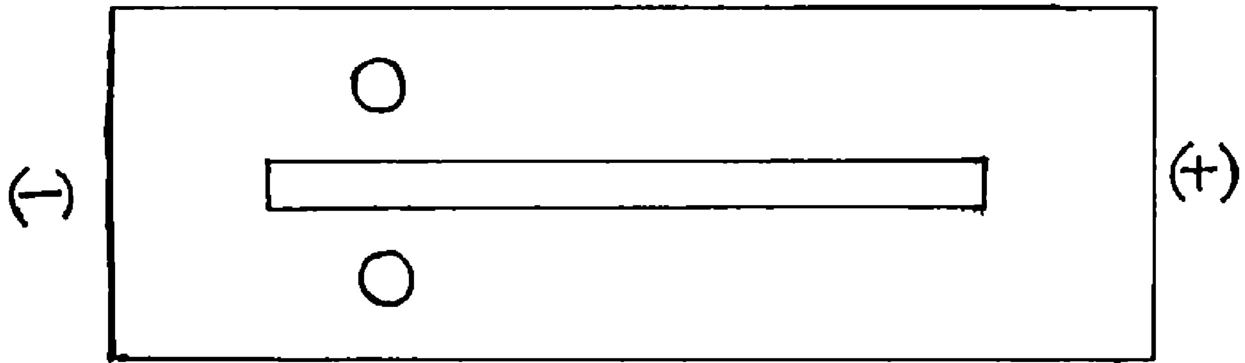


B

kept in a humid chamber and incubated in the refrigerator for 24 h during which observations were made at 8, 12, 18 and 24 h and the result recorded accordingly. The tests were repeated for different meat samples from cattle and buffalo.

3.2.11 Immunoelectrophoresis

Immunoelectrophoretic analysis was carried out as per the method described by Carpenter (1975). For this, 3 ml of 0.65 per cent agarose in barbital buffer was flooded on pre-coated microscope slide. When the agarose got solidified, wells and trough were cut as shown in Fig. 2. Wells were filled up with test antigens with 22 microlitre/ well. The slides were then placed between the buffer reservoirs with the polarity as indicated. The terminal end was connected to the buffer solution by means of filter paper strip moistened with the buffer covering about 0.5 cm of the gel on the slide. The equipment was allowed to run for 15 min at 160 volts and 9.4 mA for three slides. Then the wells were charged with antigens and allowed to run for 5 h. Soon after, the gels in the trough were removed and filled with the particular antiserum. The slides were then placed in the humid chamber and incubated in the refrigerator for 24 h and observations were made at 18 to 24 h and the results were recorded. Both unabsorbed and absorbed antisera were tested against cattle meat extract (CME) and buffalo meat extract (BME)



3 2 12 Staining

The slides subjected to immunoelectrophoresis and AGID were stained using 0 2% coomassie brilliant blue R 250 as described by Wintero <u>et</u> <u>al</u> (1990)

3 2 13 Filter paper as antigen carrier, the elution and testing

Small pieces of filter papers (5 x 1 cm/piece) were completely soaked in the meat extract from cattle (CME) and buffalo (BME) seperately The soaked filter papers were allowed to dry at room temperature and then resoaked completely in the meat extract and left overnight at room temperature to dry The dried filter papers were stored in sterile test tubes at room temperature for further use Elution of meat extract (antigen) from the filter paper was done by three types of eluants namely (i) 0 85% Sodium chloride solution (ii) Phosphate buffered saline (PBS), and (iii) Phosphate buffered saline-tween-80 (PBS T)

The elution procedure

To elute the antigen from the filter paper, two filter papers were cut into small pieces and transfered into a test tube containing 0 75 ml eluant, shaken vigorously and kept at 37°C for 30 min and then at refrigeration temperature for

2 h Then the filter papers were removed with the help of clean forceps and the eluates were centrifuged for 20 min at 5000 rpm The supernatant was collected separately in clean vials and tested against unabsorbed and absorbed antisera of cattle and buffalo by AGID In this way the filter paper eluates of CMF and BME, were eluted by three different eluants and tested on day 5 10 15 20 and 30

Results

RESULTS

4 1 Standardization of the experiment

4 1 1 Test Bleeding

RACS from Group A [RACS(A)] gave a precipitin line against CME and BME by 8 h and the intensity and number of lines developed were increased by 24 h

RACS from Group B [RACS(B)] gave positive result against CME and BME by 8 h The faint precipitin line developed became clearer and increased in number by 24 h

RABS obtained against freeze dried BME [RABS(M)] from Group C gave a weak but visible precipitin line to both CME and BME by 18 h which became slightly clearer by 24 h RABS obtained against normal buffalo serum [RABS(S)] gave positive reaction to CME and BME by 8 h and the precipitin line became clearer and denser by 24 h

Fresh sera of Cattle, buffalo and goat as antigen, gave similar reaction to all the above antisera

4 1 2 Final bleeding

Table I represents summary of AGID result on unabsorbed and absorbed antisera against various antigens

Table 1 AGID with unabsorbed and absorbed RACS and RABS

ANTISERA	ANTIGENS		
	CME	BME	GME
(A) UNABSORBED			
RACS (A)	+++	+++	++
RACS (B)	++	++	+
RABS (S)	+++	+++	++
RABS (M)	+	+	N/A
(B) ABSORBED			
RACS(A)+GFD	++	++	-
RACS(A)+SFD	++	++	-
RACS(B)+GFD	+	+	-
RACS(B)+SFD	+	+	-
RABS(S)+GFD	+	++	-
RABS(S)+SFD	+	++	-
RABS(M)+GFD	+	+	-
RABS(M)+SFD	+	+	-
RACS(A)+BFD	-	-	-
RACS(B)+BFD	-	-	-
RABS(S)+CFD	-	+	-
RABS(S)+CFD+GFD	-	+	-
RABS(M)+CFD	-	-	-
RABS(M)+CFD+GFD	-	-	-
RABS(M)+FD-GME	+	+	-

Note +++ = Strong reaction
++ = Moderate
+ = Weak
NA = Not applicable
- = Negative

4 1 2 1 Unabsorbed antisera

RACS(A) gave very strong reaction to CME and BME by 8 h Reaction to sera of cattle, sheep, buffalo and goat as antigens were also similar

RACS(B) gave moderately strong reaction to CME and BME as well as against the sera of cattle, buffalo, goat and sheep

RABS(M) gave faint precipitin line to CME and BME by 12 h which became clearer by 18 h Reaction to serum antigens of cattle buffalo, sheep and goat were similar RABS(S) developed strong precipitin lines to CME and BME by 8 h as well as to serum antigens of cattle, buffalo, sheep and goat

4 1 2 2 Absorbed Antisera

RACS(A) absorbed with GFD gave positive reaction to cattle and buffalo antigens only BY 8-12 h, a clearly visible line was formed and by 18-24 h, the intensity and number of lines formed were increased RACS(A) absorbed with SFD gave positive reaction to cattle and buffalo antigens only

RACS(B) absorbed with GFD gave positive reaction to cattle and buffalo antigens only A faint line was formed

by 12 h which became slightly clearer by 18 h RACS (B) absorbed with SFD gave similar reaction as that of RACS(B) absorbed with GFD

RABS(S) absorbed with GFD gave positive reaction to cattle and buffalo antigens only Against BME, by 12 h one clear line and a faint second line were visible, but in the case of CME only one faint line was visible By 24 h the lines developed became denser and clearer The second line developed against BME was proximal to the antiserum well RABS(S) absorbed with SFD produced a similar reaction

RABS(M) absorbed with GFD developed a very faint precipitin line against CME and BME and these lines became clearer by 24 h RABS(M) absorbed with SFD gave similar reaction to CME and BME

RACS(A) and RACS(B) absorbed with BFD did not develop precipitin line with BME and CME

RABS(S) absorbed with CFD developed positive reaction to BME and serum of buffalo only, with the development of a faint precipitin line against BME and serum of buffalo by 12 h RABS(S) absorbed with CFD plus GFD gave positive reaction to buffalo antigens only

RABS(M) absorbed with CFD alone or in combination with GFD did not develop any detectable precipitin line against

CME and BME RABS (M), when absorbed with freeze dried GME, produced a faint precipitin line against CME and BME

4 2 Experiment Proper

4 2 1 Test bleeding

Antibody produced by individual rabbits in group 'A and B gave positive reaction to CME, BME, sera of cattle, buffalo and goat Moderately clear precipitin lines were developed by 8 h and their intensity had increased by 18-24 h

4 2 2 Final bleeding

In the final bleeding, each rabbit yielded about 20 ml of blood from which approximately 9 ml sera were obtained Table 2 and figures 3 and 4 represents the AGID result on unabsorbed RACS and RABS Table 3 and figure 5 6 and 7 represents the AGID result on absorbed RACS and RABS

4 2 2 1 Unabsorbed Rabbit Anti-Cattle sera

The RACS gave very strong positive reaction to sera and meat extracts of cattle and buffalo Reaction to sera of sheep and goat were moderately strong, and weaker to GME, PME, DME and CaME In all cases the precipitin line became visible by 8 h (Table 2 and Fig 3 and 4)

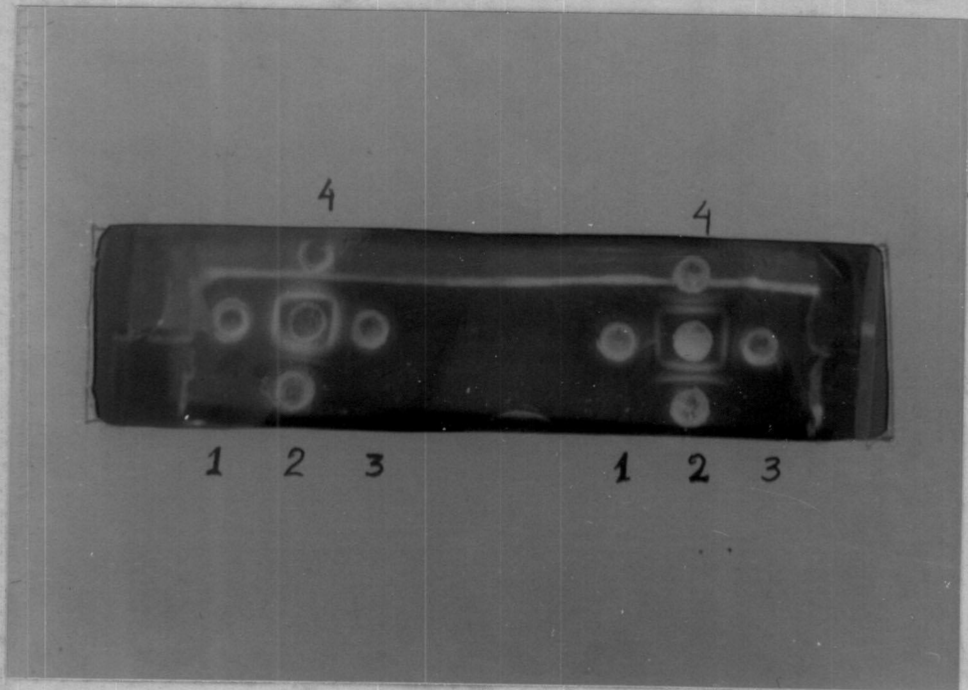
Table 2 AGID with unabsorbed RACS and RABS

Antigens	ANTISERA	
	RACS	RABS
Cattle serum	++++	+++
Buffalo serum	++++	+++
Goat serum	++	++
Sheep serum	++	++
CME	++++	+++
BME	++++	+++
GME	+	+
PME	+	+
DME	+	+
CaME	+	+

Note ++++ - Very strong reaction
+++ = Strong reaction
++ - Moderately strong reaction
+ = Weak but clear reaction

Fig.3 A: AGID with unabsorbed (a) RACS and (b) RABS, against CME and BME.

B: Diagrammatic illustration of AGID (3A). 1, 3, CME; 2, 4, BME; Centre, (a) RACS, (b) RABS.



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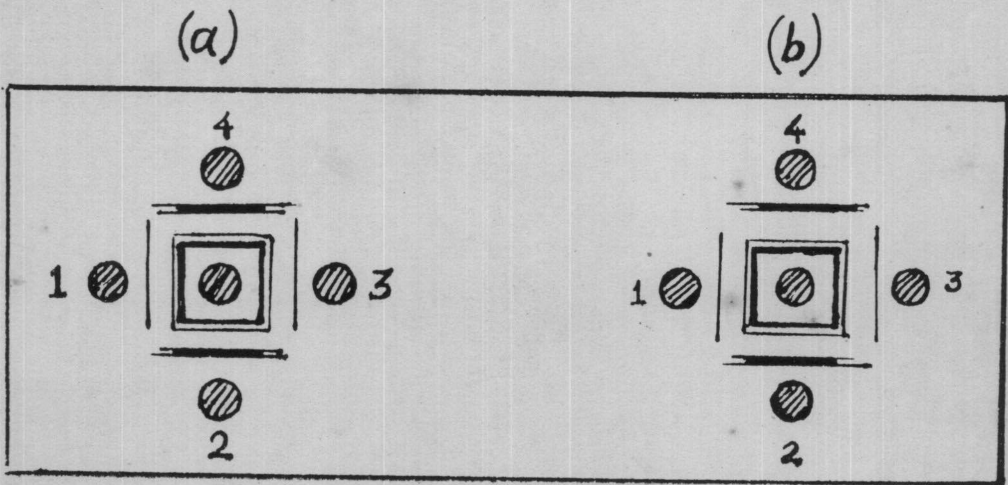
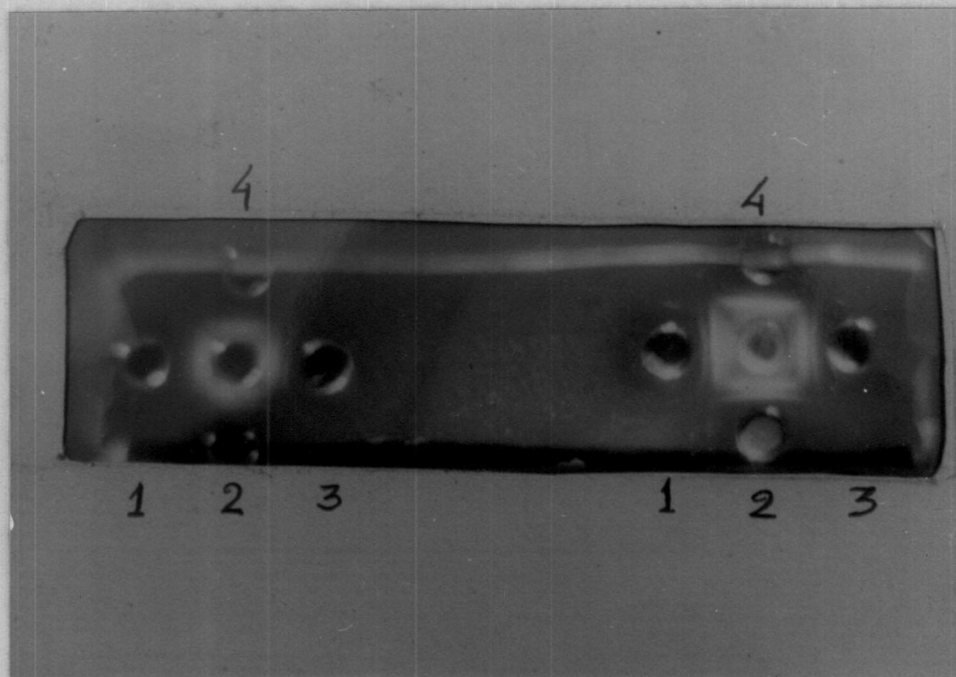


B

Fig.4 A: AGID with unabsorbed (a) RACS and (b) RABS, against sera of cattle, buffalo, sheep and goat.

B: Diagrammatic illustration of AGID (4A). 1. Cattle serum, 2. Sheep serum, 3. Goat serum and 4. Buffalo serum. Centre, (a) RACS, (b) RABS.



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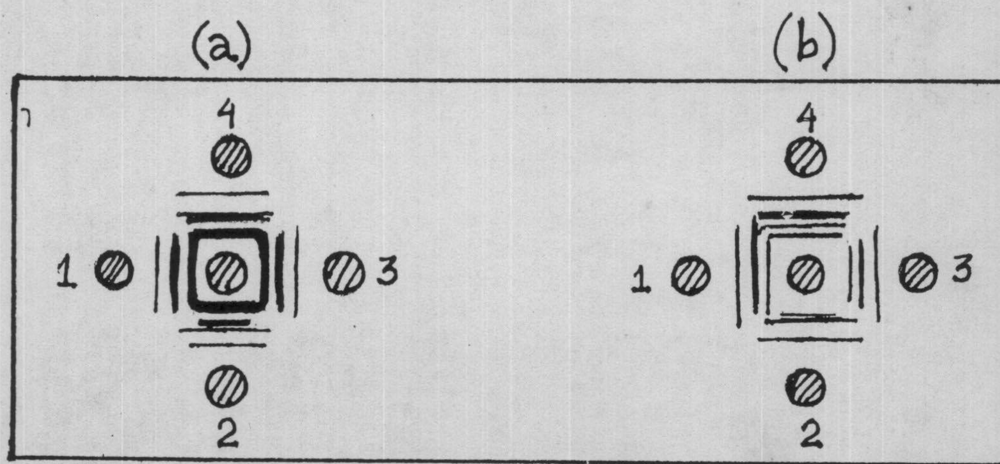


B

4 2 2 2 **Unabsorbed Rabbit Anti-Buffalo sera**

The RABS gave strong precipitin lines against sera and meat extracts of cattle and buffalo The reaction to sera of sheep and goat were moderately strong, and weaker against GME, PME, DME and CaME by 8 h (Table 2, Fig 3 and 4)

4 2 2 3 **Absorbed Rabbit Anti-cattle**

RACS absorbed with GFD gave precipitating reaction to CME, BME and sera of cattle and buffalo by 8 h There was no reaction against sera of sheep, goat, GME, PME, DME and CaME (Table 3 and Fig 5)

RACS absorbed with BFD gave positive reaction to CME and serum of cattle only by 12 h but it was negative to buffalo, goat, sheep, pig, deer and camel antigens RACS absorbed with BFD plus GFD gave negative result to all above antigens (Table 3 and Fig 6 and 7).

4 2 2 4 **Absorbed Rabbit Anti-Buffalo sera**

RABS absorbed with GFD gave precipitating reaction to BME and CME, and sera of buffalo and cattle Against BME, by 12 h one clear and a faint second line were visible but in the case of CME, only one faint line was visible by that time By 24 h, the lines developed became denser and clearer The second line developed against BME was proximal

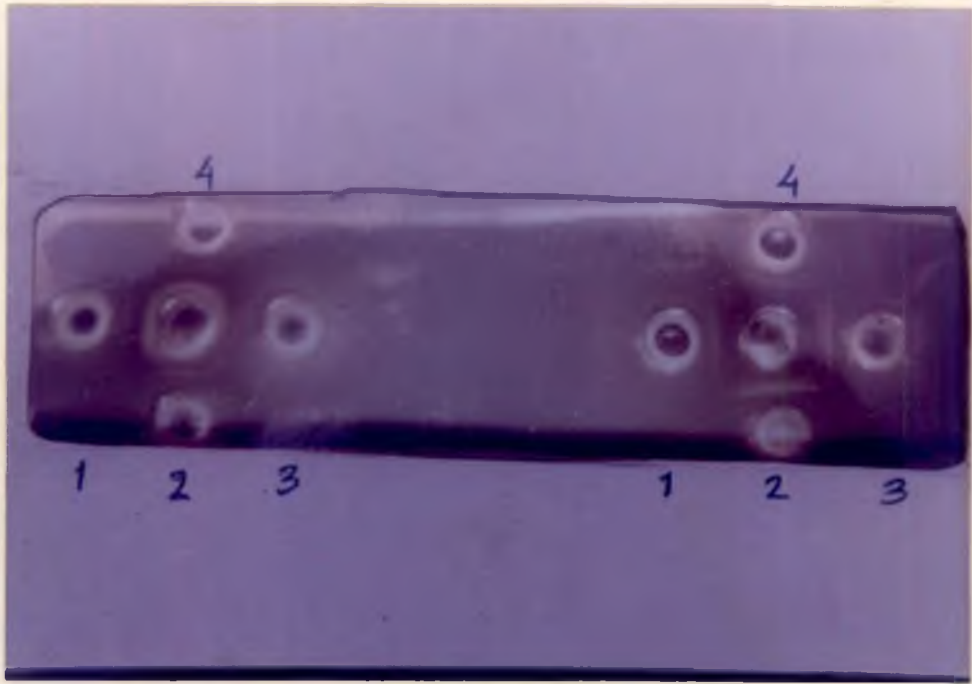
Table 3 AGID with absorbed RACS and RABS

ANTIGENS	ANTISERA					
	RACS +GFD	RABS +GFD	RACS +BFD	RABS +CFD	RACS+ BFD+GFD	RABS+ CFD+GFD
Cattle serum	++	+	+	-	-	-
Buffalo serum	+	+	-	+	-	+
Goat serum	-	-	-		-	-
Sheep serum	-		-	-	-	-
CME	+	+	+	-	-	-
BME		++	-	+	-	+
CMF		-	-	-		-
PME	-	-	-	-	-	
DME	-	-		-	-	-
CaME	-	-				-

Note + = Two precipitation lines
= One precipitation line
\- = Negative result

Fig.5 A: AGID with (a) RACS absorbed with GFD and (b) RABS absorbed with GFD against CME and BME

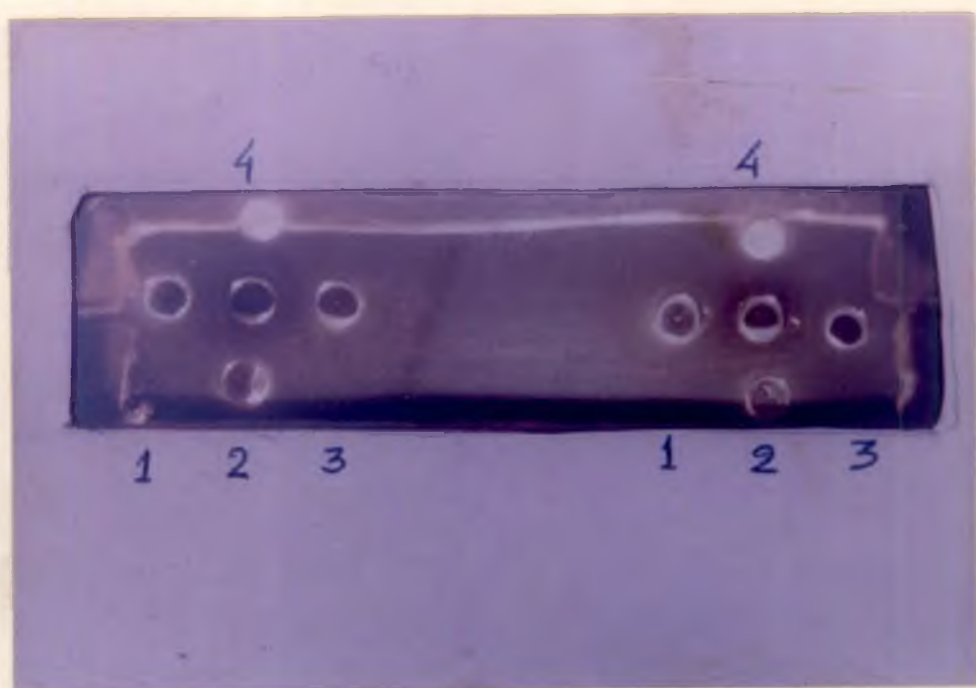
B: Diagrammatic illustration of AGID (5A) 1, 4, CME; 2, 3, BME; Centre, (a) RACS + GFD (b) RABS + GFD.



A

Fig.6 A: AGID with (a) RACS absorbed with BFD and (b) RABS absorbed with CFD against CME and BME

B: Diagrammatic illustration of AGID (6A). 1, 4, CME; 2, 3, BME; Centre, (a) RACS + BFD, (b) RABS + CFD.

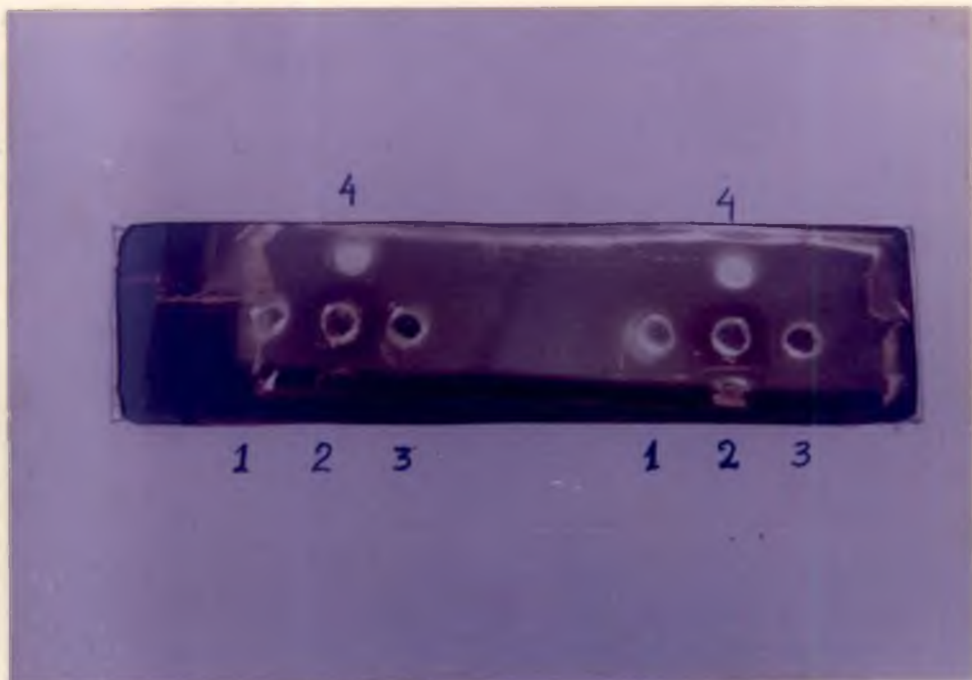


A

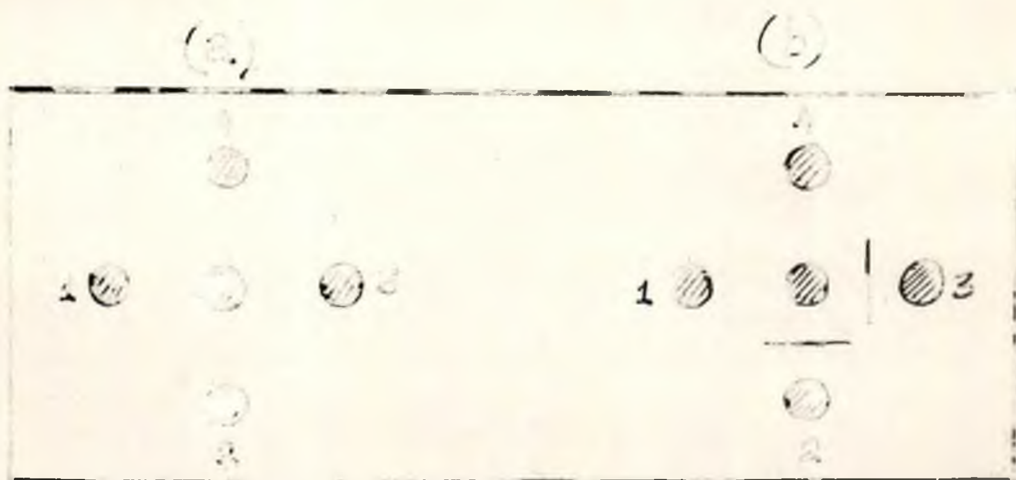
B

Fig.7 A: AGID with (a) RACS absorbed with BFD plus GFD and (b) RABS absorbed with CFD plus GFD against CME and BME.

B: Diagrammatic illustration of AGID (7A). 1, 4, CME; 2, 3, BME; Centre, (a) RACS + BFD + GFD (b) RABS + CFD + GFD.



A



B

to the antiserum well The antisera did not react against serum and meat extracts of goat, serum of sheep meat extracts of pig, deer and camel (Table 3 and Fig 5)

RABS absorbed with CFD gave positive reaction to buffalo antigens only by 8 h but were negative to other antigens even at 24 h (Table 3 and Fig 6)

RABS absorbed with CFD plus GFD gave positive reaction to buffalo serum and BME only (Table 3 and Fig 7)

4 2 2 5 **Binary meat mixtures**

RACS absorbed with BFD was tested against 20, 25, 50, 75 & 80 per cent of beef in buffalo beef and gave precipitating reactions to 25 per cent and above A faint precipitin line was developed by 12 h

RABS absorbed with CFD and RABS absorbed with CFD plus GFD were tested against 2[illegible], 25, 50, 75 & 80 per cent buffalo beef in beef and gave positive reaction to 25% and above with the development of one line by 8

4 2 2 5 [illegible]

The 10 unknown samples received for identication of species of origin, were tested against (1) RACS absorbed

Table 4 Result of AGID with blind samples

ANTIGENS (BLIND SAMPLES)	ANTISERA			Validity
	RACS+BFD	RABS+CFD	RABS+CFD+GFD	
B_1	-	+	+	100%
B_2	-	+	+	100%
B_3	-	+	+	100%
B_4	+	-	-	100%
B_5	-	+	+	100%
B_6	-	+	+	100%
B_7	-	+	+	100%
B_8	+		-	100%
B_9	+	-	-	100%
B_{10}	-	+	+	100%

Note 1 '+ indicates positive

2 - indicates negative

3 $B_1, B_2, B_3, B_5, B_6, B_7, B_{10}$ were buffalo beef samples

4 B_4, B_8, and B_9 were beef samples

with BFD, (ii) RABS absorbed with CFD, and (iii) RABS absorbed with CFD plus GFD and the results are shown in table 4

4 2 3 Immuno-electrophoresis

Results of immuno-electrophoretic analysis on various antisera against CME and BME are shown in Table 5 and Fig 8 and 9

4 2 3 1 Unabsorbed Rabbit anti-cattle and anti-buffalo sera

Unabsorbed RACS gave strong precipitating reaction to both CME and BME by 18 h Unabsorbed RABS g[illegible] weak precipitating reaction to CME and BME BY 18 h (Table 5 and Fig 8)

4 2 3 2 Absorbed Rabbit anti-cattle and anti-buffalo sera

RACS absorbed with GFD gave one thick and clear precipitin arc to both CME and BME and a faint additional arc to CME which was proximal to the antigenic well (Table 5 and Fig 9)

RABS absorbed with GFD gave a faintly spreading arc to BME and CME

RACS absorbed with BFD gave two faint precipitin arcs to CME near the antigenic well by 18 h and none to BME

Table 5 Immunoelectrophoresis with RACS and RABS

ANTISERA	ANTIGENS	
	CME (Cattle)	BME (Buffalo)
RACS	+++	++++
RABS	++	++
RACS+GFD	+	+
RABS+GFD		W
RACS+BFD	++	-
RABS+CFD	-	-
RACS+BFD+GFD	-	-
RABS+CFD+GFD	-	-

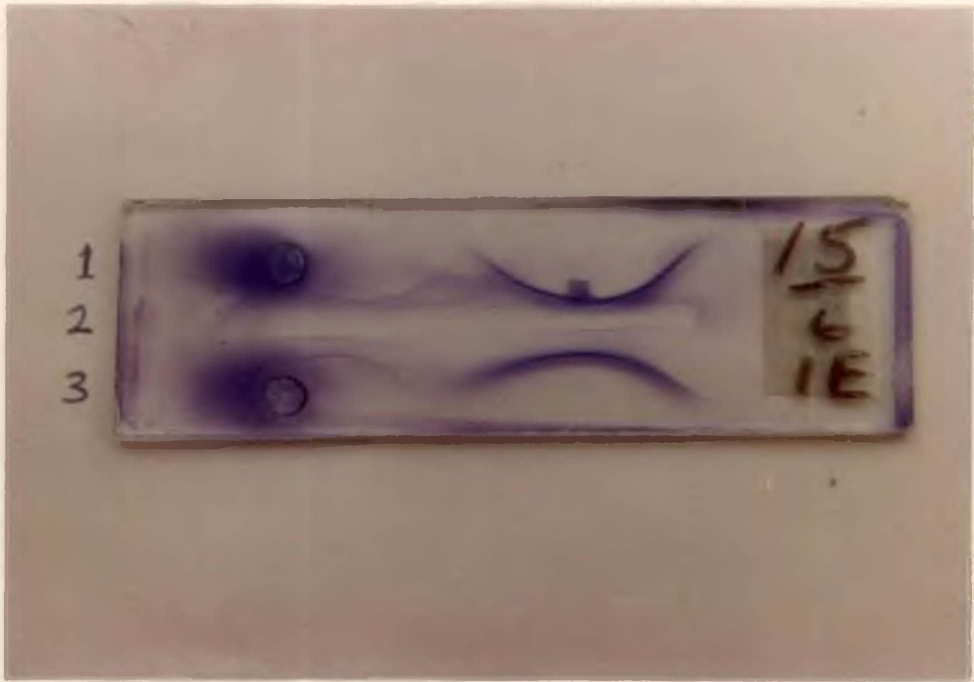
Note + indicates positive and number of precipitin arcs against the respective antigens

\- indicates negative result

W indicates weak reaction

Fig.8 A: Immunoelectrophoresis with unabsorbed RACS against CME and BME.

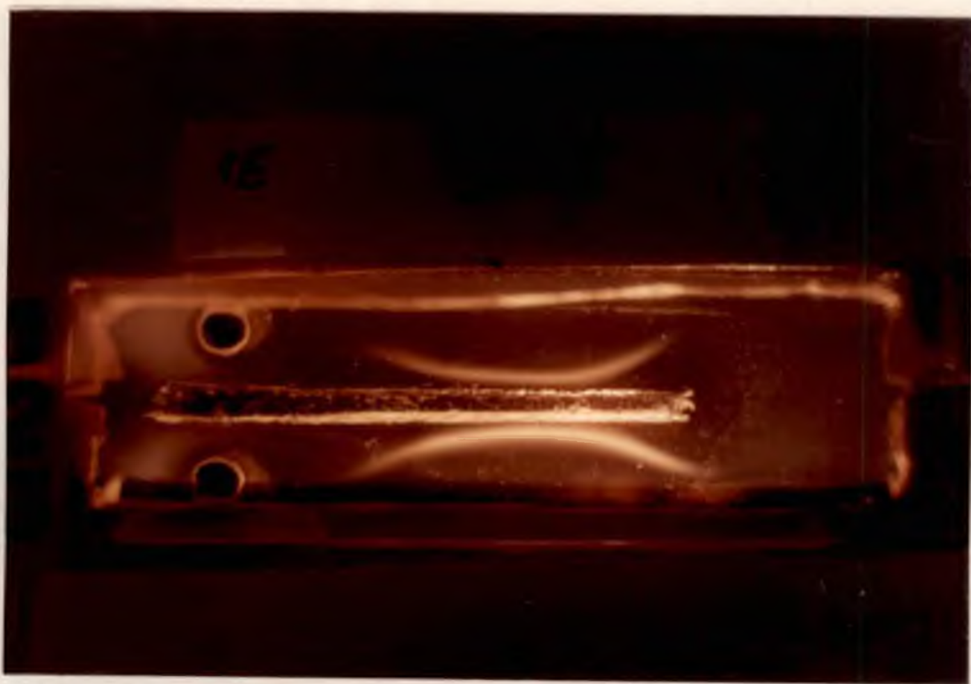
B: Diagrammatic illustration of Immunoelectrophoretogram (8A). 1, BME; 2, RACS; 3, CME.



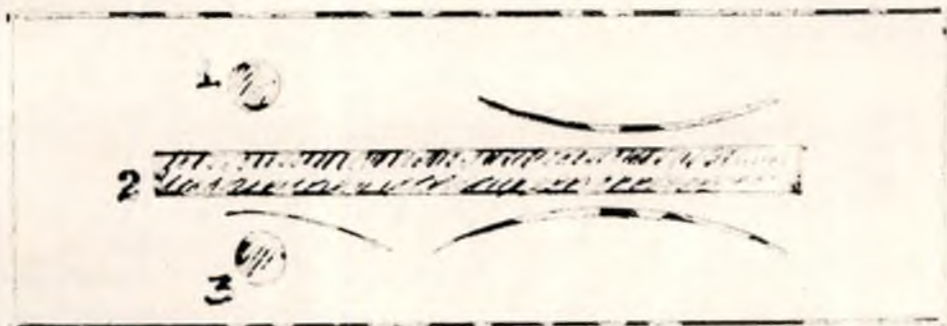
A

Fig.9 A: Immunoelectrophoresis with RACS absorbed with GFD against CME and BME.

B: Diagrammatic illustration of Immunoelectro-phoretogram (9A). 1, BME; 2, RACS + GFD, 3, CME



A



B

RABS absorbed with CFD developed a very thin precipitating arc to BME only

RACS absorbed with BFD plus GFD and RABS absorbed with CFD plus GFD did not develop any precip tin arc against BME and CME

4 2 4 **Filter paper as carrier of antigens**

All the eluates of CME and BME, prepared from 5, 10, 15, 20 and 30 days stored filter papers, gave strong reaction to unabsorbed RACS and RABS

[illegible] paper eluates of CME and BME gave moderately strong reaction against RACS absorbed with GFD and none with others (Ta[illegible]e [illegible] [illegible] 10 [illegible])

Table 6 AGID results on filter paper eluates of CME and BME

ANTISERA	DAYS*	Filter paper CME eluted by			Filter Paper BME eluted by		
		NaCl (0 85%)	PBS	PBS-T	NaCl (0 85%)	PBS	PBS-T
RACS (unabsorbed)	5	++	++	++	++	++	++
	10	++	++	++	++		++
	15	++	++	++	++	+	++
	20	++	++	++	++	+	+
	30	++	++	++	++	++	++
RABS (unabsorbed)	5	++	++	++	++	++	++
	10	++	++	++	++	++	++
	15	++	++	++	++	+	++
	20	++	++	++	++	++	++
	30	++	++	++	++	+	++
RACS+GFD	5	+	+	+	+		+
	10	+	+	+	+	+	+
	15	+	+	+	+		+
	20	+	+	+			+
	30		+	+	+		+
RABS+GFD	5	-	-	-	W	W	W
	10		-	-	-	-	
	15	-	-	-	-	-	
	20		-	-	-	-	-
	30	-	-	-	-	-	
RACS+BFD	5	W	W	W	-	-	-
	10	W	W	W	-	-	
	15	W	W	W	-	-	
	20	W	W	W	-	-	
	30	W	W	W	-	-	
RABS+CFD	5	-	-	-	-	-	
	10	-	-	-	-	-	-
	15	-	-	-		-	-
	20	-		-	-	-	
	30	-	-	-	-	-	
RABS+CFD+GFD	5	-	-			-	
	10	-	-		-	-	
	15	-	-	-	-	-	-
	20	-	-	-	-	-	
	30	-	-	-	-	-	-

Note * 'Days' = Day on which elution and testing were done from preparation of Filter papers

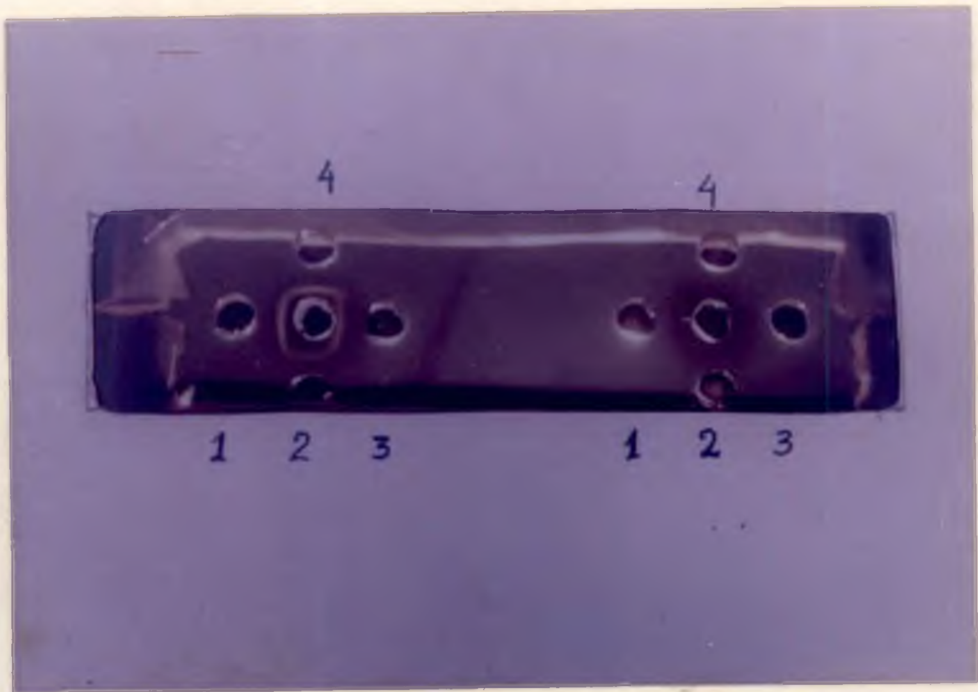
++ = Strong reaction giving more than one line

\+ = Moderately strong and one line

W = Weak reaction

Fig.10 A: AGID with filter paper eluates of CME and BME against (a) RACS absorbed with GFD and (b) RABS absorbed with GFD.

B: Diagrammatic illustration of AGID (10A). 1, 4, CME; 2, 3, BME; Centre, (a) RACS + GFD (b) RABS + GFD.



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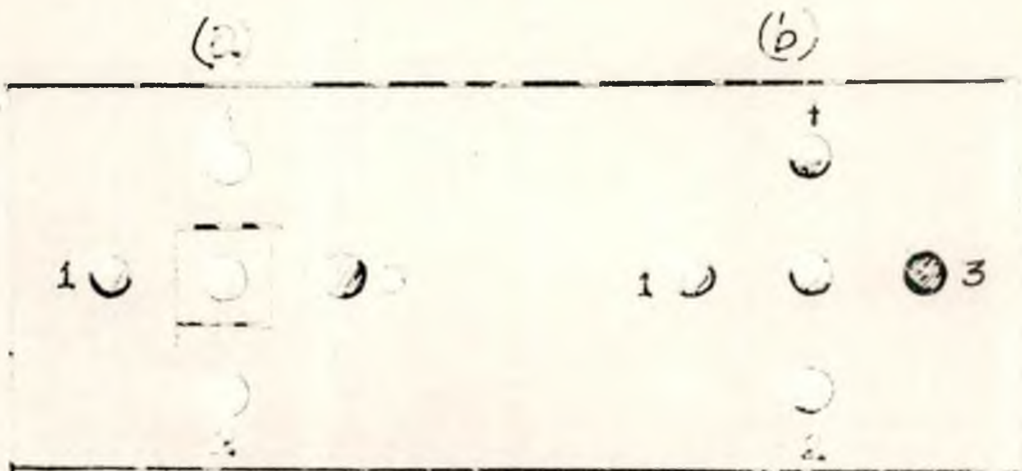
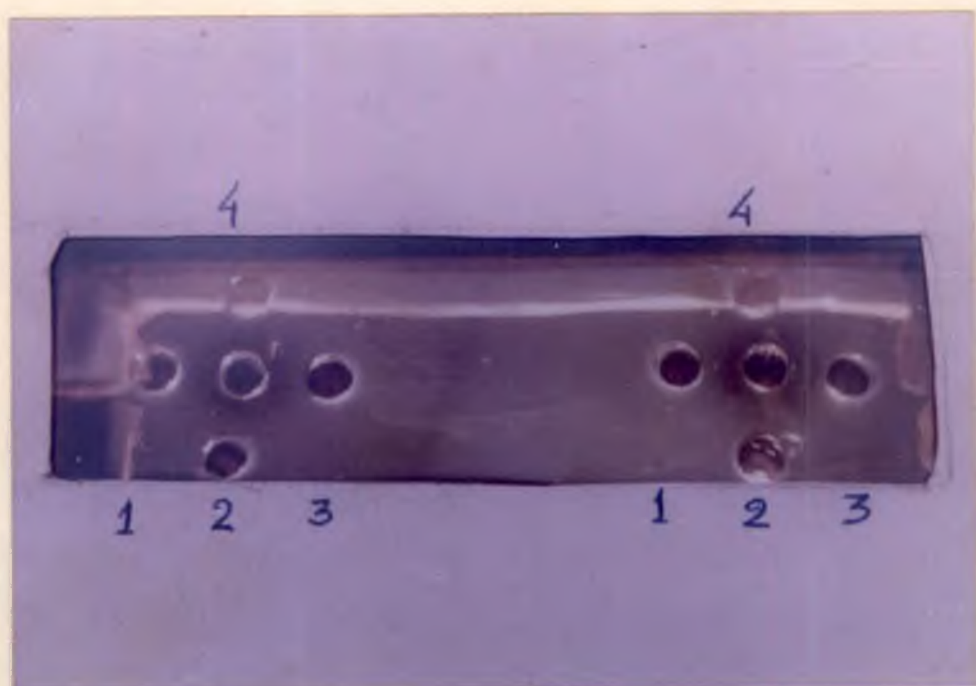


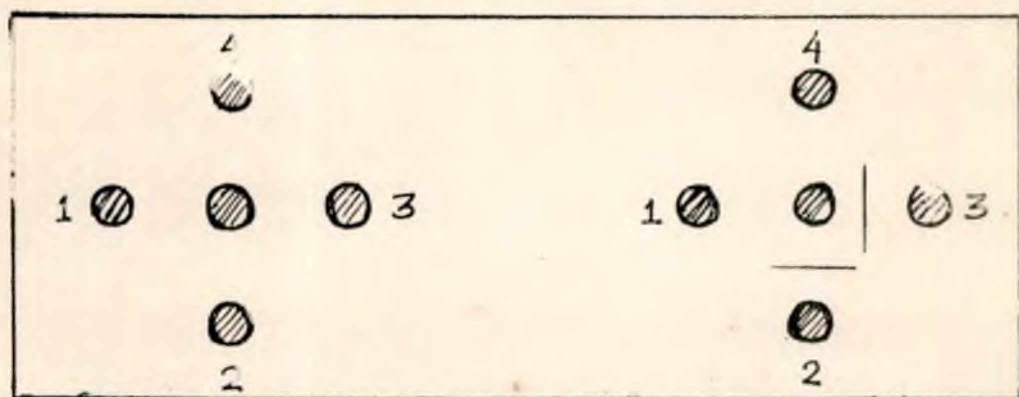
B

Fig.11 A: AGID with filter paper eluates of [illegible] and [illegible] against (a) RABS absorbed with CFD and (b) RACS absorbed with [illegible].

B: Diagrammatic illustration of AGID (11A). 1, 4, [illegible]; 2, 3, [illegible]; [illegible] (a) RABS + [illegible] (b) [illegible] +[illegible].



A



B

Discussion

DISCUSSION

5 1 Standardization of the experiment

5 1 1 Test bleeding

RACS(A), RACS(B), RABS(M) and RABS(S) produced precipitin lines against sera and meat extracts of cattle and buffalo as well as against serum of goat, indicating the development of antibody in all the rabbits The result also clearly indicated the development of cross-reacting antibodies against the closely related animals The precipitin lines developed against RACS(A) and RABS(S) were thick and dense whereas the precipitin lines developed against RACS(B) were thin and less dense The precipitin lines developed against RABS(M) were very thin and very faint The probable reason for weak and faint reaction in the case of RACS(B) may be due to heat denaturation of certain protein components in the serum immunogen The probable reason for weak and delayed reaction in the case of RABS(M) may be due to the low antigenicity of the meat extract as immunogen The difference in antibody level observed within rabbits from group A and B can also be attributed to the individual variation in responding against immunogens

5 1 2 Final Bleeding

5 1 2 1 Unabsorbed antisera

Unabsorbed antisera from each group gave similar reaction as in the case of test bled antisera but the time taken for noting an observable reaction was less and the precipitin lines developed were thicker and denser due to the increase in the antibody content This finding is in agreement with that of Proom (1943), Weitz (1952), Omland (1963a) and Muraschi et al (1965) who have reported that the more the number of injections given, the greater the activity of the antisera produced However there was no demonstrable difference between the antisera obtained after 4th injection and 10th injection with regard to species specificity This is contradictory to the report of Omland (1963a), Muraschi et al (1965) and Rodkey and Freeman (1970) who have stated that early bleedings generally resulted in the production of sera with high specificity For the production of species-specific antisera, it has been found that there was no added advantage in using heated serum and limiting the number of injection to four Compared to serum, meat extract was found to be a weaker immunogen This finding is in agreement with that of Patterson et al (1984) and Nanu et al (1984)

5 1 2 2 Absorbed antisera

Absorption of RACS(A) and RACS(B) with GFD resulted into the removal of cross reacting antibodies to sheep and goat, but not of buffalo RACS(A) or RACS(B) absorbed with SFD gave similar results indicating that the GFD and SFD were not fully effective in removing cross-reacting antibodies to make antisera monospecific

RABS(S) absorbed with GFD resulted in the removal of cross-reacting antibodies to sheep and goat and gave two precipitin lines to BME and only one against CME Thus by observing the number of lines developed, it is possible to indentify buffalo meat from beef RABS(M) absorbed with GFD or SFD gave similar reaction to both CME and BME indicating that this method was not useful in making the antiserum monospecific

RABS(S) absorbed with CFD and RABS(S) absorbed with CFD plus GFD reacted only against BME, while RACS(A) and RACS(B) absorbed with BFD did not develop any precipitin line against CME and BME This result has shown that absorption of RABS(S) with CFD alone or in combination with GFD is useful in identifying buffalo beef from beef RABS(M) absorbed with CFD alone or in combination with GFD did not react against antigens to CME and BME

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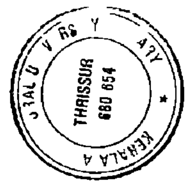

From the standardization of the experiment, the tentative conclusions made were - (i) Serum was a better immunogen than meat extract (ii) Absorption of RABS with GFD, CFD or a combination of GFD and CFD made the antisera monospecific to buffalo Hence for confirmation, the experiment is repeated with serum as immunogen, and BFD, CFD and GFD as immunoabsorbent.

5 2 Experiment Proper

As in the standardization of the experiment, on test bleeding the results showed that all the six rabbits had developed antibodies against the respective immunogens In the present study all the 12 rabbits used for the experiment had developed antibodies The result is in agreement with that of Evans (1957) Boyd (1966), Chase (1967), Crowle (1973) and Nanu _et al_ (1985) But there are reports that sometimes all the rabbits may not develop antibodies as reported by Katsube and Imaizumi (1968) and Somasekharan (1983)

5 2 1 Unabsorbed Rabbit anti-cattle and anti-buffalo sera

Unabsorbed RACS and RABS had reacted against the homologous antigens of cattle and buffalo, and heterologous antigens of buffalo, cattle sheep, goat, pig, deer and camel Such non-specific cross-reactions of antisera raised

in rabbits against a particular species serum had been widely reported by many workers (Proom, 1943, Weitz, 1952, Pinto 1961 Pandey and Pathak 1975 Swart and Wilks 1982 Doberstein and Greuel, 1985, Bansal and Mandokhot, 1988, Srinivas et al 1991) According to Weitz (1952) the production of cross-reacting antisera in rabbits by injection of a particular animal serum is due to the presence of at least two distinct groups of antigenic components namely, (i) homologous group and (ii) heterologous group Homologous group contain species specific as a major component and group specific as minor component and is responsible for the production of antibodies It is the group specific component which react with the closely related species Heterologous group is more complex in nature and is responsible for production of antibodies, reacting with the antigens of many distantly related mammals Esteves and Binaghi (1972) have also reported that there are common antigenic determinants among animals which are responsible for production of cross-reacting antibodies Batty (1984) stated that the intensity of cross-reaction in general was, proportional to their zoological relationship

5 2 2 **Absorbed Rabbit Anti-Cattle Serum**

After absorption of RACS with GFD, the cross-reacting antibodies against antigens of sheep, goat, pig, deer and

camel could be removed but not against buffalo antigens When RACS was absorbed with BFD, it could remove not only the cross reacting antibodies against the above antigens but also that of buffalo RACS absorbed with a combination of BFD and GFD caused the removal of antibodies against that of heterologous antigens and homologous antigen When GFD is used for absorption it could not remove the cross- reacting antibodies against the closely related buffalo and gave similar reaction to CME and BME, indicating that GFD could not be used for making RACS monospecific When BFD is used for absorption, the resultant antiserum reacted only against cattle antigens, thus making the RACS monospecific When a mixture of BFD and GFD were used for absorption, the resultant antiserum could not gave detectable precipitation line to any antigens, including that of cattle The probable reason for this may be due to the fact that goat is more closely related to cattle than buffalo and share common antigenic determinants with cattle as reported by Esteves ad Binaghi (1972) According to them, cow, goat and sheep share a common antigenic determinant Therefore, GFD in combination with BFD seems to remove the antibodies present in RACS to such a low level that even the homologous cattle antigen could not produce any detectable precipitin. When BFD is used, it could remove antibodies against group specific and

heterologous antigens like buffalo sheep goat, deer camel and pig When GFD is used it removed antibodies against goat, sheep deer camel and pig but not against buffalo Similar type of reaction had been reported by Doberstein and Greuel (1985) According to them anti-impala sera could be made monospecific by absorption with sera of springbok or Thomson s gazelle On saturation of anti-eland sera with calf serum, most of the cross-reacting antibodies were removed, except those against the closely related greater kudu When the saturation of anti-eland sera was done with calf serum and lyophilized kudu meat extract, the resultant anti-eland sera were not useful even against the homologous eland antigens

5 2 3 Absorbed anti-buffalo Serum

GFD as an immunoabsorbent was able to make the RABS to a useful reagent for the species identifications of meat In the case of BME there were always two precipitin lines by 24th h and only one line against CME The reason for this differential reaction may be due to the fact that goat is more closely related to cattle and share a common antigenic determinant as stated by Esteves and Binaghi (1972) CFD and, mixture of CFD and GFD as immunoabsorbent resulted in making the RABS monospecific Such absorbed antisera identified buffalo meat only CFD or, CFD plus GFD removed the cross-reacting antibodies against (a) the closely

related species like cattle, sheep and goat, (b) distantly related camel and deer, and (c) pig which do not even belong to the order <u>Ruminantia</u> The probable reason for this can be attributed to the explanation made by Weitz (1952) and Esteves and Binaghi (1972)

5 2 4 Lowest detectable level

In the case of RACS absorbed with BFD, the lowest detectable level for beef was 25 per cent in a binary mixture of buffalo meat and beef RABS absorbed with CFD and RABS absorbed with CFD plus GFD also could detect the presence of buffalo beef at a level of 25 per cent and above in a mixture of beef and buffalo beef According to Kurth and Shaw (1983), it is not possible to obtain anti-buffalo sera which consistently can detect buffalo meat at 10 per cent level Cutrufelli <u>et al</u> (1987) reported that the detectable limit of beef in the meat product is $\geq$ 10 per cent using ORBIT Sherikar <u>et al</u> (1993) reported the detectable limit of 10 per cent or above in the mixture containing cattle, buffalo sheep and goat as adulterants In the present study the detectable level was 25 per cent or more This low sensitivity may be due to various factors like low antibody content in the antisera used, or may be due to variation in antigenic content of meat extract which are controlled by residual serum albumin content (Hayden

1978), pH of meat sample during extraction, type of muscle used for antigen extraction and age of animal (Wijingaards and van Biert 1985) Besides, Swart and Wilks (1982) and Sherikar et al (1993) have reported that the detectable level for closely related species are always higher than distantly related species

5 2 5 Validity of AGID

AGID test result obtained from ten unknown samples against RACS absorbed with BFD revealed that the samples B4, B8 and B9 were beef and all of them were identified correctly With RABS absorbed with CFD and RABS absorbed with CFD plus GFD, test antigens from samples B1, B2, B3, B5 B6, B7 and B10 were identified as buffalo beef and the identifications made were correct The simultaneous use of RACS absorbed with BFD and RABS absorbed with CFD or RABS absorbed with CFD plus GFD will be able to correctly identify a given sample as either beef buffalo beef or not

5 2 6 Immunoelectrophoresis

From the outcome of the result obtained, it was not possible to identify beef or buffalo beef correctly owing to the variation in the number of precipitin arcs developed from the samples tested This may be due to various factors such as the pH of the buffer, gel strength, power fluctuation and period of run, antigenicity of the test

samples antibody content of the antisera To confirm or disprove the result obtained here further detail study is required because there are reports that by observing the number of arcs developed in immunoelectrophoretogram it is possible to identify between beef and buffalo beef (Ramadass and Misra, 1981, Aulakh et al , 1994)

5 2 7 Filter paper as carrier of antigen

The eluates of CME and BME in the eluants of physiological saline, PBS PBS-T reacted uniformly in a positive way with unabsorbed RACS and RABS The resultant reaction of RACS absorbed with GFD against CME and BME eluates were similar but weaker But in both instances, it was not possible to differentiate beef from buffalo

RABS absorbed with GFD, RACS absorbed with BFD, RABS absorbed with CFD, RABS absorbed with CFD plus GFD did not produce any reliable reaction even at 24 h

With unabsorbed antisera, use of filter paper as a carrier of antigen produced an easily recognizable precipitin but not with absorbed antisera This may be due to lower antibody content in the absorbed antisera and, or due to a minor alteration in the nature of antigen that would have taken place during the drying of the filter paper and elution The above problem may not have any significant effect when the antibody content of the

absorbed antisera is at a higher concentration Therefore further work on drying and eluting the antigens and how to increase the antibody content of the absorbed antisera will be beneficial

As an eluant for CME and BME filter paper, it was found that either NaCl PBS or PBS-T can be used without affecting the result

From the present study it is concluded that normal serum is a good immunogen, heat inactivated serum do not inhibit the development of cross-reacting antibodies, immunoabsorption of RABS is more promising than that of RACS, absorption of RABS with GFD resulted into the identification and differentiation of buffalo beef and beef, AGID is simple and reliable test, immunoelectrophoresis with absorbed and unabsorbed antisera did not produce any conclusive results, the absorbed RACS and RABS were able to detect beef and buffalo beef at a level of 25 per cent or more in binary mixtures, with unabsorbed antisera, filter paper was a good carrier for meat antigen and found to be good even after 30 days of storage, for using with absorbed antisera further improvement of filter paper technique is suggested, and, for the purpose of eluting the filter paper antigens, any one of the three eluants can be used without affecting the result

Summary

SUMMARY

Partial or total substitution of meat and meat products is rampant in the meat trade throughout the world Such malpractices are not only illegal but also unethical to the religious and social customs and from public health point of view Detection of such illegal practices in the meat industry is very important for the said reasons above and to safegaurd the interest of the consumers Substitution and adulteration of buffalo beef with beef is very common in meat export trade but the export of beef from the country is totally prohibited by the law Immunological methods has many advantages for use in the meat industry and factory laboratories for identification of species origin of meat provided the antisera against the species to be determined, is monospecific Hence, the present study was undertaken (1) To develop a suitable method to produce monospecific antibeef sera The anti-beef sera is to be raised in rabbits using fresh serum as immunogen and the cross-reacting antibody is to be removed by absorption with serum antigen from closely related species for differentiation of beef from buffalo meat by Agar gel immuno-diffusion test, and (2) To evaluate the suitability of filter paper as a carrier for meat antigen from the field

Based on the results from the standardization of the experiment, six rabbits, three in each group, were immunised with pooled sera of cattle and buffalo The rabbits were injected intramuscularly @ 1 ml per animal for 8 - times at the interval of five days, Rabbit anti-cattle serum (RACS) harvested at the terminal bleeding was pooled and divided in to four parts One part was kept as such unabsorbed, and the other three parts were absorbed with (i) GFD, (ii) BFD and (iii) BFD plus GFD respectively The rabbit anti-bufalo serum (RABS) harvested at the terminal bleeding was treated in a similar way except that instead of BFD, CFD was used for absorption The unabsorbed and absorbed RACS and RABS were tested by AGID against CME, BME, GME, PME, DME, CaME and Sera of cattle buffalo, sheep and goat The absorbed and unabsorbed antisera were also tested by immuno electrophoresis against CME and BME Blind samples and binary mixture of beef in buffalo beef and buffalo beef in beef in the proportion of 20, 25, 50, 75 and 80 were also tested with the absorbed antisera by AGID

To investigate the feasibility of the filter paper as carrier of meat antigen for storing and transporting from one place to another, pieces of filter papers were soaked in cattle meat extract (CME) and buffalo meat extracts (BME) separately The filter papers were dried and resoaked and dried The dried filter papers were eluted and tested after storage of 5, 10, 15, 20 and 30 days by AGID For

evaluating the utility of various eluants physiological saline (NaCl 0 85 %), phosphate buffered saline and phosphate buffered saline - tween 80 were used for eluting the antigen from the dried filter paper

The results could be summed up and concluded as follows -

* Blood serum is a better immunogen than meat extract

* Heat inactivation of the serum did not preclude the production of cross reacting antibodies

* The unabsorbed RACS and RABS strongly reacted to the homologous cattle and buffalo antigens, cross reacted to (a) the closely related buffalo and cattle, (b) distantly related sheep and goat, (c) more distantly related deer and camel, and (d) to very distantly related pig

* RACS absorbed with BFD and RABS absorbed with CFD became monospecific to cattle and buffalo antigens respectively

* Immunoabsorption of anti-buffalo serum is more promising RABS absorbed with GFD is capable of identifying both beef and buffalo beef which is not possible with any other absorbed antisera Confirmation of the result is also possible with CFD absorbed RABS, if a contingency arise

* Findings of the standardization test and the experiment proper are contradictory in the case of Rabbit anti-cattle sera BFD absorbed RACS may or may not be useful in identifying beef Further confimatory work is required to prove or disprove the result.

* The minimum detectable level of adulteration with either beef or buffalo beef is 25 per cent The monospecific RACS and RABS could identify the origin of 10 unknown samples correctly

* Filter paper is a good medium for carrying meat antigen to be used later with unabsorbed antiserum

* NaCl, PBS and PBS-T are equally good eluants for elution of the filter papers

* Eluates of BME or CME did not elicit any specific reaction with absorbed antisera It will be very useful to find out the reason and its remedy, if any

* AGID is a simple and reliable test for identifying species origin of meat

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DEVELOPMENT OF MONOSPECIFIC ANTI-BEEF SERA

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ABSTRACT OF A THESIS

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ABSTRACT

Agar gel immunodiffusion is a simple and reliable test for identifying the species origin of meat, provided the antisera to be used are monospecific. A study was undertaken to make Rabbit anti-cattle serum (RACS) and Rabbit anti-buffalo serum (RABS) monospecific by absorption with the freeze dried sera of goat (GFD), buffalo (BFD), cattle (CFD) and a combination of GFD and CFD or GFD and BFD. Though it was found that the RACS was made monospecific by absorption with BFD, production of monospecific RABS through absorption with GFD or CFD, is more desirable. Absorption of RABS with GFD alone enabled to identify both beef and buffalo meat samples which can be further confirmed by RABS absorbed with CFD. RACS absorbed with BFD and RABS absorbed with CFD could identify a level of 25 per cent or above adulteration with beef and buffalo beef respectively.

Filter paper was found to be good carrier of beef and buffalo meat extract antigens and storing it for upto 30 days did not influence the test result with unabsorbed antisera. All the three eluants, NaCl, PBS and PBS-T were found to be equally useful for elution of the meat antigen from the dried filter paper.

APPENDIX

1 Barbital buffer (after K K Sarin, 1983)

Stock solution A 0 2 M solution of sodium barbitone (mol wt 206 18)

Sodium barbitone	42 2 gm
Distilled water	1000 0 ml

Stock solution B 0 2 M Hydrochloric acid (35% GR, mol wt 36 46)

Hydrochloric acid	18 ml
Distilled water	1000 ml

Working solution (pH 8 2)

Stock solution	A	50 ml
Stock solution	B	12 7 ml
Distilled water		137 3 ml

2 Phosphate buffered saline tween 80 solution (PBS-T) (after Huang et al 1988)

Sodium phosphate dibasic dihydrate[1] (Na_2 HPO_4 $2H_2O$	2 9 g
Sodium chloride (NaCl)	8 0 g
Potassium phosphate monobasic (KH_2PO_4)	0 2 g
Potassium chloride (KCl)	0 2 g
Tween - 80[2] (0 05%)	0 5 ml
Distilled water	1000 0 ml

3 Phosphate buffered saline (PBS)

Sodium phosphate, dibasic, dihydrate[1] (Na_2 HPO_4 $2H_2O$	2 9 g
Sodium chloride (NaCl)	8 0 g
Potassium phosphate, monobasic (KH_2PO_4)	0 2 g
Potassium chloride (KCl)	0 2 g
Distilled water	1000 0 ml

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(1) Orginally -dodecahydrate (Na_2HPO_4 $12H_2O$)

(2) Orginally Tween -20

4 Staining solution (After Wintero *et al* 1990)

Coomassie brilliant blue R250 (0 2%)	0 4 g
Ethanol (44 %)	88 ml
Acetic acid (10%)	20 ml
Distilled water (46%)	92 ml

5 Destaining solution (Wintero *et al* , 1990)

Ethanol (44 %)	88 ml
Acetic acid (10%)	20 ml
Distilled water (46%)	92 ml